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■Full-Length Paper■

Functional Analyses of a Neural Cell Specific Variant of Microtubule-Associated Protein 4

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Abstract: The present study was conducted to analyze the functions of a recently reported neural cell specific variant of MAP4 (MAP4-SP) that lacks 72 consecutive amino acid residues in a region that is rich in proline and basic residues (pro-rich region). Although our previous study (Matsushima *et al.*, 2005)[12)], using the microtubule-binding domains of the isoform and wild type MAP4 (MAP4-LP), demonstrated a difference in the microtubule bundling activity of the two proteins, here, using the full-length forms of the MAP4 proteins, we show that the proteins do not differ in their microtubule bundling activity both *in vitro* and *in vivo*. Expression of the MAP4 proteins, as C-terminal fusions to green fluorescent protein (GFP), in neuroblastoma cells revealed that MAP4-SP decorated microtubules were more remarkable in appearance than MAP4-LP decorated microtubules in the neuronal growth cones. Moreover, a microtubule destabilizing protein, septin2, which interacts with the pro-rich region of MAP4, was more active in destabilizing MAP4-SP-microtubules than MAP4-LP-microtubules *in vitro*. The susceptibility of MAP4-SP microtubules to destabilization by septin could be attributed to the weaker binding affinity of MAP4-SP for microtubules, as was reported earlier. Taken together, the current findings suggest the possibility that the neural MAP4, with its short pro-rich region, could be important in maintaining more dynamic microtubules in neural cells, and thus allowing more plasticity in and rapid morphological changes of these cells.

Key Words: microtubule, microtubule-associated protein 4 (MAP4), pro–rich region, neural MAP4, microtubule bundling, septin2, microtubule stability and dynamics

Introduction

While the brain microtubule-associated proteins (MAPs)* such as MAP1, MAP2 and tau were the first MAPs to be studied in great detail, subsequent studies on MAPs from tissues other than the brain, led to the discovery of a new, ubiquitous class of MAP referred to as 'microtubule associated protein 4' (MAP4)[1)]. MAP4 shares many structural and functional properties with the neuronal MAPs, MAP2 and Tau. They are all heat stable, promote the polymerization of tubulin, and stabilize microtubules *in vitro*. In the intracellular environment, MAP4 is believed to play important roles in the organization and dynamics of both interphase and mitotic microtubules[2-4)]. MAP4 proteins are composed of an

*Abbreviations: MAP, microtubule-associated protein; LP, long pro-rich; SP, short pro-rich; GFP, green fluorescent protein; AP, assembly promoting; MTB, microtubule binding; PJ, projection; DMEM, Dulbecco's modified eagle medium; FCS, fetal calf serum; PCR, polymerase chain reaction; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; FPLC, fast performance liquid chromatography; RB, reassembly buffer; MES, 2-(N-morpholino) ethanesulfonic acid; EGTA, Ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; DIC, differential interference-contrast; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

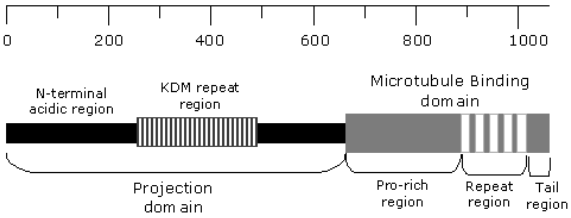

Fig. 1. Schematic diagram of the primary structure of MAP4.

N-terminal acidic domain and a C-terminal basic domain. The basic C - terminal domain binds to acidic microtubules, and is known as the microtubule-binding domain. The microtubule-binding domain of MAP4 is highly conserved among the MAP4 proteins from different species and is also partly homologous to the microtubule- binding region of the brain MAPs, MAP2 and tau[5,6].

The microtubule-binding domain of MAP4 is divided into three sub-domains (Fig.1): 1) *Pro-rich region:* a region rich in proline and basic residues that was suggested to promote the nucleation of microtubule assembly *in vitro* by bridging proto-filaments laterally[7,8]. 2) *Repeat region*: a region containing 3-5 imperfect repeats of assembly promoting (AP) sequences that was shown to be essential for microtubule assembly and suggested to serve as a microtubule elongating factor[7,8]. 3) *Tail region:* a short hydrophobic C-terminal sequence which does not bind to microtubules, but was thought to be necessary for proper microtubule assembly[7]. The N-terminal acidic domain of MAP4 was termed the 'projection domain' because this domain does not bind to microtubules and protrudes from the microtubule wall as projections. The projection domain of MAP4 has been suggested to suppress the microtubule bundling activity of the microtubule-binding domain and maintain spacing between the microtubules[9].

Differential regulation of the intracellular dynamics and organization of the microtubule cytoskeleton by different isoforms of MAPs is believed to play a key role in cell growth and morphogenesis[10]. To date, four types of MAP4 cDNAs have been identified that differ from each other in the number or arrangement of the AP sequences[11]. In addition, we have identified a neural cell specific variant of MAP4 from bovine adrenal medulla that lacks 72 consecutive amino acid residues in the pro-rich region, from amino acid position 649-721, of wild type MAP4[12]. The missing region comprises a complete exon of wild type MAP4, and is possibly generated by an alternative splicing. Because of the short pro-rich region of the isoform, it was termed 'MAP4-SP', and the ubiquitous MAP4, with a long (full-length) pro-rich region, was termed 'MAP4-LP'. The missing region of MAP4-SP is highly conserved among mammalian species and a similar variant was also detected in rat cells[12]. In either species, expression of the isoform is restricted to neural-ectoderm derived tissues such as the brain and adrenal medulla. *In vitro* analyses using bacterially expressed truncated fragments containing the microtubule binding domains of MAP4-LP and MAP4-SP revealed minor differ ences in their microtubule binding and assembly promoting activities, except that the *in vitro* reconstituted microtubules in the presence of the MAP4-SP fragment were single microtubules instead of microtubule bundles induced by the MAP4-LP fragment[12].

To date, only a few proteins reportedly interact with MAP4 *in vivo*, and most of them are involved in the phosphorylation of MAP4. Very recently, Kremer *et al.*[13] reported that mammalian septins bind to MAP4 and thus regulate the stability of microtubules. Septins are a family of GTP binding proteins that play important roles in cytokinesis. There are about 12 proteins in this family, which forms oligomers and are often found in association with microtubules and the actin cytoskeleton *in vivo*[13]. An extensive search for septin binding partners led the authors to reveal an interaction between MAP4 and septin. The interaction occurs through the binding of septin2 to the pro-rich region of MAP4, which blocks the interaction of MAP4 with microtubules, reducing microtubule stability *in vivo*. These findings raise the possibility that the neural specific MAP4 isoform, which we have described, may differ in its ability to interact with the septins because of the short pro-rich region. A variation in the binding affinity between MAP4 and septin could have distinct effects on the cellular microtubule dynamics and organization.

In our previous study[12], the most prominent functional difference between the neural variant (MAP4-SP) and wild type (MAP4-LP) MAP4 was observed in their *in vitro* microtubule bundling

activity. These analyses were, however, carried out using only the microtubule binding domains of the corresponding proteins, and it is not known whether the proteins possess similar properties in their full-length forms. In this paper, we demonstrate that the two proteins do not differ in their microtubule bundling activity both *in vitro* and *in vivo*, but differ in their ability to organize microtubules in neuronal growth cones. Moreover, a difference was observed in the septin-mediated regulation of microtubules, assembled *in vitro*, in the presence of MAP4-LP and MAP4-SP, suggesting that neural MAP4 may differentially regulate microtubules in a tissue specific manner.

Materials and Methods

Materials

The bacterial expression vector for full-length mouse septin2 (pT7-HS-Sept2) was kindly provided by Brandon Kremer (Center for Cell Signaling, Department of Microbiology, University of Virginia School of Medicine, Charlottesville, VA, USA). Rabbit anti-MAP4 antiserum was obtained as described by Kotani *et al.*[14]. Tubulin was purified by phosphocellulose column chromatography, from a twice-cycled porcine brain microtubule protein fraction, as described previously[15,16]. MAP4 fragments containing the microtubule binding domains of MAP4-LP and MAP4-SP were purified as decribed earlier[10]. Other reagents used in the study were of reagent grade unless otherwise mentioned.

Cell culture

Mouse neuroblastoma cell line, NG 108-15 cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin at 37.5°C in 5% CO_2. For live cell observation, NG 108-15 cells were cultured on cover slips coated with poly-L-lysine (0.01%) and laminine (12.5 μg/ml).

Construction of expression plasmids

Because cDNA clones for full-length MAP4 proteins were not found in our previous search[12], bacterial and mammalian expression vectors that encompass full-length MAP4 were constructed from shorter constructs, expressing the projection (PJ) domain, and the microtubule-binding domains of MAP4-LP (MTB-LP) and MAP4-SP (MTB- SP). The C-terminal part of the PJ domain and the N-terminal parts of both MTB domains share a common region (Fig.2). DNA encoding the PJ domain and the MTB domains were first amplified by PCR using primers as indicated in Fig. 2. The primer sequences were: P1-F, 5′-gaagc tagcctcagtcttgc agatgcg-3′; P1-R, 5′-cacgactgcttctg gtga-3′; P2-F, 5′-ccagtcaaagacatggctc-3′; and P2-R, 5′-agaagctta gatgcttgtctcctggatc-3′. The PCR pro- ducts were gel purified and the PCR products for PJ and MTB-LP or PJ and MTB-SP were mixed in separate reaction mixtures with dNTPs and taq DNA polymerase (Takara Bio Inc., Shiga, Japan) without the addition of primers. The reaction mixtures were then subjected to 30 cycles of the thermal reaction so that the common region at the junction of PJ and MTB domains acts as a primer to complete the synthesis of the full-length MAP4 sequences. Each thermal cycle consisted of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 3 min, respectively. One μl of these reaction products were then subjected to PCR using P1-F and P2-R primers to amplify full-length MAP4 encoding sequences. To construct bacterial expression vectors, primers P1-F and P2-R were designed to contain the restriction endonclease sites N*he*I and *Hind*III, respectively. The full-length MAP4 inserts were ligated in frame to the *Nhe*I/*Hind*III sites of the expression plasmid vector pET21d(+) (Stratagene, La Jolla, CA) to generate pET-MAP4-LP and pET-MAP4-SP plasmids. Mammalian expression vectors were also constructed by employing the same strategy except that primer P1-F was designed to contain

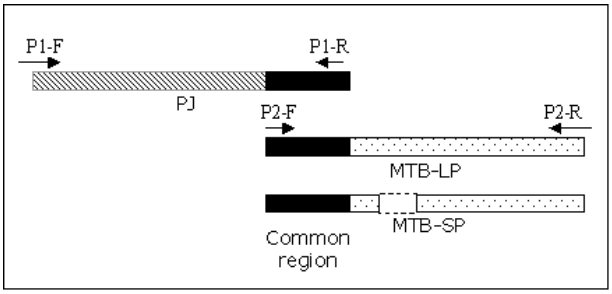

Fig. 2. Schematic diagram of the MAP4 cDNAs used to construct the expression vectors of full-length MAP4-LP and MAP4-SP. Arrows indicate the location of different primers. Diagram not drawn to scale.

a *Bgl*II site instead of the *Nhe*I site. The inserts were ligated in frame to the *Bgl*II/*Hind*III sites of pEGFP-C3 (Clontech, CA, USA) to express MAP4 genes as fusions to the C-terminus of the green fluorescent protein (GFP). To construct the expression vectors of MAP4 proteins fused with other fluorescent proteins, such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), the inserts expressing CFP and YFP were first cut from pECFP-N1 and pEYFP-C1 plasmids (Clontech, CA, USA), respectively, at *Age*I and *Bsr*G1 sites. The inserts were then ligated to the *Age*I/*Bsr*G1 sites of pEGFP-C3 to replace GFP with CFP and YFP, generating pECFP-C3 and pEYFP-C3 plasmids, respectively. The full length MAP4-LP and MAP4-SP inserts were then cut from the previous GFP constructs at *Bgl*II/ *Hind*III sites and ligated to the same sites of pECFP-C3 and pEYFP-C3 plasmids to express CFP-MAP4-LP and YFP- MAP4- SP, respectively.

Purification of full-length MAP4-LP and MAP4-SP

Bacterial expression of MAP4 proteins was induced by isopropyl-1-thio-β-D-galactopyrano -side (IPTG) (Takara Bio Inc., Shiga, Japan) to a final concentration of 0.5 mM in LB medium (10 g/liter NaCl, 10 g/liter bactotryptone, 5 g/liter yeast extract, pH 7.2) containing chloramphenicol (25 μg/ml) and ampicillin (150 μg/ml) at 37°C for 3-4 hours. Cells were collected by centrifugation and resuspended in 20 MEM and heat-treated directly in a boiling water bath for 8-min. The cell debris was removed by centrifugation and purification of the MAP4 proteins from the heat stable cell extracts was carried out using a prepacked, high capacity ion exchange column, Econo-Pac High S cartridge (Bio-Rad, Hercules, CA) coupled to a FPLC system (Amersham Biosciences, Uppsala, Sweden) according to the instruction manual.

Purification of septin2

The expression of septin2 was induced by the addition of IPTG (Takara Shujo, Tokyo, Japan) to a final concentration of 0.5 mM and incubation at 18°C overnight. Cells were collected by centrifugation and dissolved in 8 ml native binding buffer (50 mM NaH_2PO_4, 0.5 M NaCl, pH 8.0). Cells were lysed by one freeze thaw cycle at –80°C and sonication. Cell debris was removed by centrifugation and the supernatant was transferred to a fresh tube. His tagged septin2 from the soluble fraction was then purified by ProBond™ Nickel chelating resin (Invitrogen life technologies, CA, USA) according to the instruction manual.

***In vitro* polymerization of tubulin**

For the turbidity assay, tubulin (10 μM) was added to reaction mixtures containing only reassembly buffer (RB: 100 mM MES, pH 6.8, 0.1 mM EGTA, and 0.5 mM $MgCl_2$), MAP4-LP (0.8 mg/ml) in reassembly buffer, or MAP4-SP (0.8 mg/ml) in reassembly buffer. GTP was present in all reactions at a final concentration of 0.5 mM. Tubulin polymerization was monitored by measuring the increase in absorbance at 350 nm at 37°C in a UV spectrophotometer (U 2000, Hitachi, Tokyo, Japan) for up to 30 min. Microtubule cosedimentation assays using septin2 and MAP4 fragments were carried out under the same reaction conditions. Briefly, purified septin2 was added to reaction mixtures containing tubulin and MAP4 fragments to final concentrations of 10 μM and 2 μM, respectively, in a reassembly buffer containing 0.5 mM GTP. The mixtures were then incubated at 37°C for 30 min and centrifuged at 16 000 × *g* for 30 min. The pellets were resuspended in the same volume of RB, and both the supernatants and pellets were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel.

Electron microscopy

Microtubule samples were prepared as in the turbidity assay described above, and mounted on 300-mesh carbon coated copper grids. The grids were left for 1 min, blotted and negatively stained with 0.75% uranyl acetate before observation under a Philips Tecnai F20-FEG microscope, operating at 120 kV.

Transfection and live cell observation

NG 108-15 cells were transfected with 1μg of constructs using SuperFect transfection reagent (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions. Transfected cells grown on laminin-coated cover glasses were mounted on an open heating chember (Warner Instruments,

Hamden, CT) operating at 37°C. Approximately 1 ml of pre-warmed growth medium was poured onto the cover glass and the chamber was mounted on a confocal laser- scanning microscope (Zeiss LSM510; Carl Zeiss GmbH., Jena, Germany) for live cell observation. All images were analyzed by ImageJ software (NIH, USA).

Results

Microtubule bundling activity of full-length MAP4 proteins

We reported earlier that the MAP4-LP fragment containing the microtubule-binding domain formed microtubule bundles *in vitro*, whereas the corresponding fragment of MAP4-SP did not. The difference in the microtubule bundling activities of MAP4 fragments was demonstrated by the significant variation in the turbidity values of microtubules, as well as by electron microscopy[12]. In this study, we aimed to investigate whether these proteins behave similarly in full-length forms. Although the purified fractions of full-length MAP4 proteins used in this study contained a large number of degradation products of MAP4 (data not shown), it was expected that the presence of the full-length forms should exert their effects in spite the presence of their degradation products. Therefore, the partially purified, full-length MAP4 protein preparations were used for a turbidity based microtubule bundling assay. Both proteins induced microtubule assembly *in vitro*, as revealed by the significantly higher turbidity values of tubulin preparations, containing full-length MAP4-LP or MAP4-SP, than the control preparation that lacked MAP4 proteins (Fig. 3A). However, in contrast to our previous observations on MAP4 fragments[10], the turbidity values of preparations containing full-length MAP4 proteins were almost identical (Fig. 3A), suggesting that none of the proteins induced microtubule bundles. The turbidity data were further confirmed by the fact that no microtubule bundles were found by electron microscopy (Fig.3B). The MAP4 proteins, therefore, do not differ in their microtubule bundling activity, *in vitro*, in full-length forms.

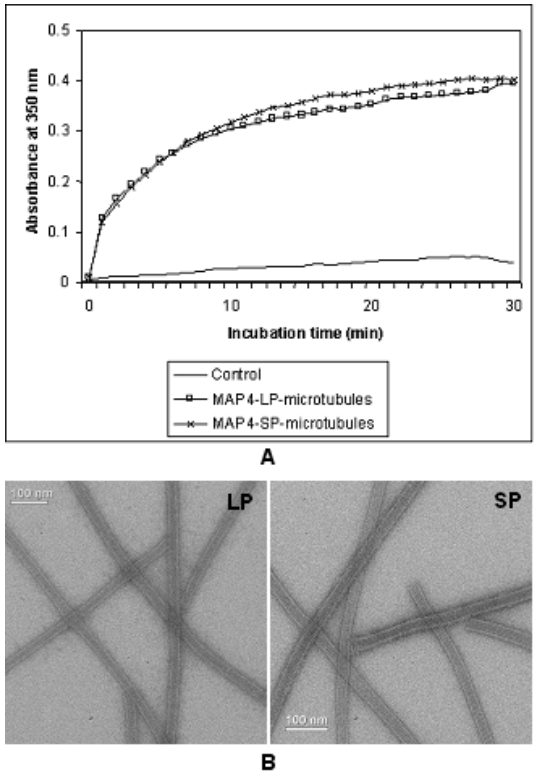

Fig. 3. Turbidometric and electron microscopic analyses of microtubules assembled *in vitro*, using the full-length forms of MAP4-LP and MAP4-SP. (A) Tubulin (10 μM) was mixed with purified MAP4-LP or MAP4-SP (0.8 mg/ml) in reassembly buffer, and polymerization was initiated by raising the temperature from 0℃ to 37℃ (see Materials and Methods). Control experiment was carried out in the same way without MAP4 proteins. (B) Microtubule assembly was induced by MAP4-LP or MAP4-SP *in vitro*. The samples were mounted on carbon-coated grids and negatively stained for electron microscopy.

Microtubule organization in cells expressing GFP-MAP4-LP and GFP-MAP4-SP

With an aim to study the intracellular behavior of the neural specific isoform of MAP4 as compared to wild type MAP4, GFP-MAP4-LP and GFP-MAP4-SP were expressed in a mouse neuroblastoma cell line, NG 108-15 cells. In cell biology research, GFP and other fluorescent proteins are now being extensively used as reliable and effective fluorescent reporters of many proteins of interest that directly show the intracellular localization of the target protein in living cells. In our study, GFP was tagged to the N-terminal end of full-length MAP4 proteins, so that GFP did not interfere with the microtubule binding activity of the C-terminal microtubule-binding domain. Once expressed in mammalian cells, we found that both MAP4 proteins had a typical filamen-

tous distribution in the cytoplasm, indicating their localization with microtubules (Figs. 4A and 4B). Further evidence regarding the microtubular localization of the MAP4 proteins came from the fact that their filamentous distributions were disrupted by treatment of the cells with the microtubular inhibitor, nocodazole (Figs. 4C and 4D). These results indicate that the expressed proteins were functionally active *in vivo*, and thus the staining pattern of GFP, reports about the microtubule organization of the MAP4 expressing cells.

The general organization of microtubules was the same in cells expressing MAP4-LP or MAP4-SP (Figs. 4A and 4B), and in a co-expressed cell, both MAP4 proteins decorated the same microtubules throughout the cell (shown by open arrows in Figs. 4C and 4D). However, when individually expressed, the MAP4-SP decorated microtubules were found to be more remarkable in appearance in the neuronal growth cones than the MAP4-LP decorated microtubules (shown by closed arrows in Figs. 2E and 2F). This feature was more clearly noticeable in well-developed growth cones after considerable elongation of neurites. To investigate whether MAP4-SP could induce process formation in non-neuronal cells, as was reported for MAP2 and tau[17,18], rat fibroblastic cell line, 3Y1 cells were transfected with the GFP-MAP4-SP construct. However, no process formation was observed in MAP4-SP expressing cells and microtubule organizations in these cells were similar to those expressing MAP4-LP (data not shown). These data indicate that unlike MAP2 and tau, MAP4-SP itself is not capable of forming processes in non-neuronal cells.

Interactions between MAP4 proteins and septin 2

Septin 2, one of the mammalian septins, was recently reported to bind to the pro-rich region of MAP4 and thus inhibit the MAP4 activity to bind, stabilize and bundle microtubules *in vitro*[13].
Because MAP4-SP lacks a significant portion of the pro-rich region, we wanted to investigate whether this protein differs with MAP4-LP, which possesses the full-length pro-rich region, in its ability to interact with septin2. To investigate this possibility, bacterially expressed septin2 was added to reaction mixtures containing tubulin and

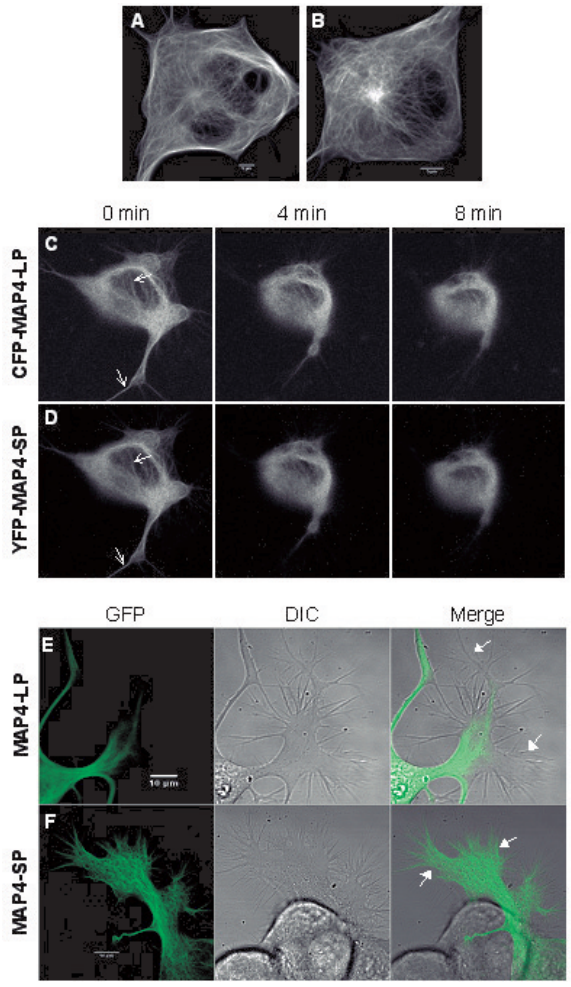

Fig. 4. Live cell observation of cells expressing GFP-MAP4-LP or GFP-MAP4-SP. (A,B) NG 108-15 cells were transfected with GFP-MAP4-LP construct (A) or the GFP-MAP4-SP construct (B) and observed as described in the Materials and Methods. (C, D) NG 108-15 cells were co-transfected with CFP-MAP4-LP (C) and YFP-MAP4-SP (D) constructs. Cells were prepared for live observation as described in the Materials and Methods and mounted on a confocal laser-scanning microscope (Zeiss LSM 510). The microscope is equipped to detect cyan fluorescence from CFP and yellow fluorescence from YFP at the same time from the same cell, using lasers of different wavelengths. Nocodazole was added to a final concentration of 10 mg/ml during live cell observation, and the effect of nocodazole was monitored by capturing images at 30 sec intervals for a total of 10 min. (E,F) NG 108-15 cells were transfected with GFP-MAP4-LP (E) or GFP-MAP4-SP (F) constructs and observed as before. Left panel: GFP staining shows the MAP4-decorated microtubule distribution. Middle panel: Differential interference-contrast (DIC) images of the cells showing cell shapes. Right panel: Merged images showing the intracellular localization of MAP4 proteins.

MAP4 fragments, possessing the microtubule binding domains of MAP4-LP or MAP4-SP, under microtubule assembly conditions. The effects of septin2 on the assembly promoting and microtubule binding activity of MAP4 proteins were then analyzed by the amounts of precipitated tubulin and co-precipitated MAP4 proteins, respectively, as revealed by SDS-PAGE. Figure 5 (lanes 1 and 2) demonstrates that the addition of septin2 had no effect on the assembly promoting and microtubule binding activity of MAP4-LP. However, the amount of precipitated tubulin was reduced in the presence of septin2, when MAP4-SP was used to assemble microtubules *in vitro* (Fig. 5, lanes 3P and 4P). Moreover, in the presence of septin2, a small amount of MAP4-SP remained in the supernatant fraction, while no MAP4-SP was detectable in the supernatant fraction of the control preparation, which lacked septin2 (Fig.5, lanes 3S and 4S). These results suggest that the MAP4-SP-microtubules could be more prone to destabilization by septin2 than MAP4-LP- microtubules.

In addition to the MAP4 proteins, septin2 also appeared in the microtubule-pellet fraction (Fig. 5, lanes 2P and 4P). However, we found that septin2 was precipitated under microtubule assembly conditions even in the absence of tubulin and MAP4 proteins (data not shown). Therefore, the co-sedimentation of septin2 with microtubules is not related to its ability to interact with microtubules. These data are also consistent with the original report of Kremer *et al.* [13] on septin2.

The inhibitory effect of septin2 on the interaction of MAP4-SP with microtubules was not so prominent as that reported by Kremer *et al.* [13] concerning the effect of septin2 on the pro-rich region. This may be related to the fact that, in addition to the pro-rich domain, our MAP4 fragments contained the assembly promoting domains, which have higher binding affinities for the microtubules.

Moreover, Kremer *et al.* [13] used septin trimers (septin2:6:7) instead of only septin2, as was used in this study. We predict that the inhibitory effect of septin on the microtubule binding and assembly promoting activities of MAP4-SP could be more pronounced with the trimer.

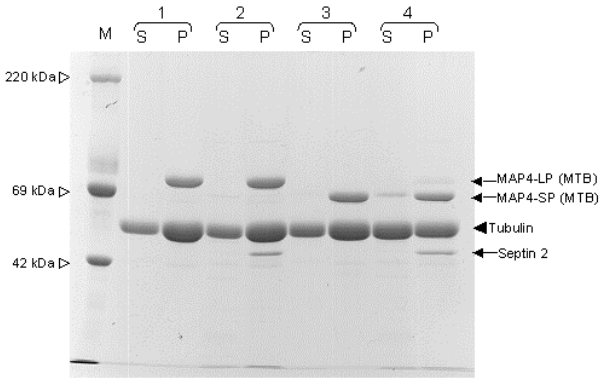

Fig. 5. Effect of septin2 on MAP4-microtubule interaction. Septin2 was added to reaction mixtures containing tubulin (15 μM) and MAP4 fragments (2 μM). Microtubules were polymerized and microtubule bound and unbound fractions were analyzed by SDS-PAGE. Lanes 1 and 3 contained control preparations of MAP4 LP and MAP4-SP, respectively, without septin2; Lanes 2 and 4 contained MAP4-LP/Septin2 and MAP4-SP /Septin2, respectively. S: Supernatant; P: pellet.

Discussion

The main focus of the present study was to functionally characterize the neural variant of MAP4, MAP4-SP, which lacks 72 consecutive amino acid residues in the N-terminal portion of the pro-rich region. Because many functions of the ubiquitously distributed MAP4, MAP4-LP that contains the intact pro-rich region, are known, analyses were carried out in terms of comparisons between the two proteins. In our previous report[12], we showed that MAP4-SP shares many properties with MAP4-LP, but differs in its microtubule bundling efficiency. Here, using the full-length forms of these proteins, we have shown that neither of the two proteins induces microtubule bundles *in vitro*. This is consistent with the widely accepted hypothesis that the projection domain of MAPs acts to space microtubules, which was demonstrated in the case of MAP4 by Iida *et al.*[9]. However, the present data do not exclude the possibility that the deletion in the pro-rich region causes a change in the tertiary structure of MAP4-SP at the junction of the projection domain and the microtubule-binding domain, and thus MAP4-LP and MAP4-SP could maintain different spacing between microtubules, as we have discussed elsewhere[12]. Further investigations using more purified MAP4 proteins are necessary to address this issue.

Next, to investigate the effect of MAP4 proteins

on the organization of microtubules, we expressed these proteins in NG 108-15 cells. GFP-MAP4 proteins were localized to microtubules, as revealed by their filamentous distribution in the cytoplasm and by the disruption of their filamentous staining pattern upon treatment of cells with the microtubular inhibitor, nocodazole (Fig.4). Overexpression of MAP4 proteins did not cause any significant change in the microtubule organization, and the general staining patterns were the same for both MAP4-LP and MAP-SP. However, it was interesting to note that the MAP4-SP decorated microtubules were more remarkable in the neuronal growth cones than the MAP4-LP decorated ones. This observation supports our previous speculation that MAP4-SP could have a neural cell-specific role.

During neuronal development, the coordinated reorganization of microtubules and action filaments is believed to play a key role in the formation of growth cones from extending lamellipodia, but little is known about the mechanisms of such coordinated behavior. Previously, MAP2 was suggested to be a candidate that rearranges the cytoskeleton, during neuronal growth and differentiation, through its ability to interact with both microtubules and actin filaments[19]. Since MAP4 proteins share many structural features with MAP2 in their microtubule -binding domains, it seems possible that MAP4-SP might play a similar role in the neuronal growth cones. The predominance of MAP4-SP compared to MAP4- LP in the actin rich growth cones (Fig. 4E and 4F) also supports this idea.

Recently, Kremer *et al.*[13] demonstrated that a small G protein, septin2, interacts with the pro-rich region of MAP4 and thus destabilizes microtubules *in vivo*. Because MAP4-SP lacks a significant portion of the pro-rich region, it was expected that the two forms of MAP4 could be differentially regulated by septin2 with respect to their microtubule binding and assembly promoting activities. Although, it was possible that MAP4-SP, with its incomplete pro-rich region, could bypass the septin-mediated regulation, the result was opposite: septin2 was more efficient in exerting its effect on the activity of MAP4-SP. We could not observe any effect of septin2 on MAP4-LP, which is consistent with the findings of Kremer *et al.*[13] that the inhibitory effect of septin on the binding of MAP4 to microtubules was limited to the pro-rich region alone, and the effect was not observed with a MAP4 fragment containing both the pro-rich region and the repeat region. The higher sensitivity of MAP4-SP microtubules to septin could be related to the weaker binding affinity of MAP4-SP for microtubules[12].

In conclusion, we have demonstrated, using the full-length proteins, that neural MAP4 shares many properties with its wild type counterpart *in vitro*, despite the lack of a highly conserved region within the prorich region. However, the two proteins showed slightly different intracellular localizations, i.e., the neural MAP4 variant was more prominent in the neuronal growth cones. Furthermore, the neural MAP4-induced microtubules were more susceptible to destabilization by septin2. These results suggest that the protein might confer dynamicity to neuronal microtubules, especially in the cell periphery, allowing remodeling of the microtubule cytoskeleton and thus providing increased plasticity to the cells during neuronal polarization.

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